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ATTACHMENT OF MATING REACTIVE *PARAMECIUM* TO POLYSTYRENE SURFACES: IV. COMPARISON OF THE ADHESIVENESS AMONG SIX SPECIES OF THE GENUS *PARAMECIUM*

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Abstract

The relationship between mating reactivity and ability of cells to attach to polystyrene Petri dishes was investigated in six species of the ciliated protozoan *Paramecium.* Mating reactive-dependent attachment to polystyrene surfaces was seen in *P. caudatum, P. multimicronucleatum, P. tetraurelia,* and *P. trichium.* Attachment was rarely seen in either mating reactive or non-reactive cells of *P. bursaria* irrespective of extracellular ion concentration, temperature, and swimming velocity. Induction of attachment occurred in mating reactive cells of *P. bursaria* by treatment with trypsin. Strong affinity of cells for polystyrene surfaces was seen in *P. duboscqui* even when they were mating non-reactive or when applied to polystyrene dishes with reduced hydrophobicity. Induction of micronuclear activation by attachment—which was found in *P. caudatum*—was not observed in attached cells of the other species.

Introduction

Attachment of cells to polystyrene surfaces is a common phenomenon widely seen in various types of cells. In the ciliated protozoan *Paramecium caudatum,* attachment differs from that in other types of cells such as bacteria, slime molds, or cells in tissue culture of higher animals, since only mating reactive cells of *P. caudatum* can attach to the bottom of polystyrene dishes (Falcon 1007) and the attachment occurs only at the tips of ventral cilia where mating reactivity is restricted (Kitamura, 1982). An increase in affinity of cells for polystyrene occurs during the initial step of conjugation, which involves specific ciliary agglutination between cells of complementary mating types (Kitamura, 1984). This attachment, which seems to involve hydrophobic interactions, also induces the first step of nuclear activation (early migration of the gametic nucleus) and the subsequent loss of mating reactivity seen in normal conjugation. Thus, a series of hydrophobic interactions on the cell surface is believed to play an important role in the conjugation process of *P. caudatum.* Similar attachment was reported in another species, *P. multimicronucleatum,* though nuclear activation was not observed (Kitamura and Steers, 1983).

Conjugation of *Paramecium* is initiated by specific cell recognition between complementary substances on the cell surface, substances which are simple, non-conjugated proteins (Metz and Butterfield, 1951; Cohen and Siegel, 1963; Kitamura and Hiwatashi, 1978). It provides a good system for studying the problems of cell surface interactions since it is an extremely simple system to the extent that the interacting cells are structurally identical, the sexual interactions are strictly cell surface events,

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Abbreviation: EMM = early micronuclear migration.

and no diffusible substances are involved (Sonneborn, 1937; Metz, 1954; Hiwatashi, 1969, 1981; Miyake, 1981; Kitamura and Hiwatashi, 1984; Hiwatashi and Kitamura, 1985).

The polystyrene-attachment phenomenon may be useful to studying the cell surface interactions of *Paramecium,* since conditions for the attachment is very simple, *i.e.,* cells attach to polystyrene surfaces in simple salt solution and the attachment is not complicated by adsorption to the substratum of serum proteins which are usually added to tissue culture media. In addition, polystyrene surfaces are known to be used for the radioactive labelling of cell surface proteins (Chin and Lanks, 1980). Therefore, one means of studying specific interacting surface substances can employ use of artificial substrata of known structure and properties (Lanks and Chin, 1982).

This study employs such an approach using polystyrene surfaces to open the way to identify the cell surface substances involved in the conjugation process of *Paramecium.* I will describe first adhesiveness of cells to polystyrene dishes in six species of *Paramecium* to determine the relationship between mating reactivity and the ability to attach to polystyrene surfaces. Light microscopical studies also were performed to examine whether micronuclear activation is induced by attachment.

Materials and Methods

Cells and culture methods

Ten stocks of syngen 3 of *P. caudatum,* 10 stocks of *P. multimicronucleatum,* syngen 2, 4 stocks of *P. tetraurelia,* 4 stocks of *P. trichium,* syngen 1, 20 stocks of *P. bursaria,* syngen 1, and 15 stocks of *P. duboscqui* were used. All the stocks of *P. multimicronucleatum* were homozygous for the acyclic allele in the expression of mating type (Barnett, 1966). Stocks of *P. multimicronucleatum* (stocks 103, 109, 203, and 204), *P. tetraurelia,* and *P. bursaria* were kindly provided by Dr. A. Barnett (University of Maryland), by Dr. S. Koizumi (Miyagi College of Education), and by Dr. H. Endoh (University of Tsukuba), respectively. Stocks of the CH-series in *P. multimicronucleatum, P. caudatum, P. trichium,* and *P. duboscqui* (original source, Dr. Shi Xin-Bai, Harbin Normal University) were obtained from the Hiwatashi collection at Tohoku University.

All the stocks were cultivated at 25°C in 1.25% (w/v) fresh lettuce juice medium diluted with Dryl's solution (Dryl, 1959) which was inoculated with *Klebsiella pneumoniae* one day before use (Hiwatashi, 1968). In cultures of all organisms except *P. bursaria,* several hundred cells were inoculated into an 18 × 180 mm test tube containing 2 ml of culture medium and expanded by adding fresh medium of 4 ml, 10 ml and 10 ml on successive days. Within one day after the last feeding the cultures reached the stationary phase of growth and most cells exhibited strong mating reactivity. The stocks of *P. bursaria* were cultured in test tubes at 25°C with a fixed illumination cycle of 12 h dark and 12 h light by adding 2 ml, 4 ml, 8 ml, and 8 ml of culture medium on successive days to reach stationary phase one day after the last feeding. After reaching the stationary phase, cultures of *P. bursaria* were left in a sunny room to maintain the diurnal rhythm of mating reactivity.

Measurement of attachment to polystyrene dishes

To measure cell attachment the previously reported method (Kitamura, 1982) was used. Cells were washed twice by centrifugation in a hand-operated centrifuge with a standard saline solution (1 m*M* KCl, 1 m*M* $CaCl_2$, and 1 m*M* Tris-HCl, pH 7.1). The washed cells were equilibrated in glass test tubes with the same solution

TABLE I

Attachment of cells to the surface of polystyrene Petri dishes in P. caudatum, *syngen 3*

Stocks	Mating type	% Cells attached to the dishes[a]	
		Non-reactive cells[b]	Mating reactive cells
27aG3	V	3 (1–5)	79 (73–83)
Yt3-G1	V	2 (0.5–4)	80 (74–85)
StG1	V	1 (0.1–2)	67 (61–74)
Yt3-G9	VI	2 (0.5–4)	91 (87–94)
27aG3-6	VI	2 (0.5–4)	66 (60–72)
C103	VI	2 (0.5–4)	78 (72–84)
CHB	VI	0 (0–2)	21 (16–27)
CHB-s2	VI	0 (0–2)	23 (16–30)
CHB-s4	VI	0 (0–2)	9 (5–15)
CHB-s5	VI	0 (0–2)	18 (13–22)

[a] Cell suspensions of each stock in a medium that contained 1 m*M* KCl, 1 m*M* $CaCl_2$, and 1 m*M* Tris-HCl, pH 7.1, were introduced into polystyrene dishes. Two minutes later, photographs were taken to measure attached cells. Numbers in parentheses show 90% confidence limits.

[b] Logarithmically growing cells in sexually mature period.

for 10 min at 21°C, after which 5 ml of the cell suspension containing about 1000 cells/ml was added to a 50 mm disposable polystyrene Petri dish (Falcon 1007). After 2 min the test dishes were photographed with a 1 s exposure and the percentage of cell attachment to the polystyrene surface was measured by counting between 100 and 200 cell tracks in each dish. The swimming velocity of cells was determined by measuring the length of about 20 tracks of photographs taken with a 2 s exposure. Cells measured for swimming velocity were placed in dishes for tissue-culture (Falcon 3002), in which attachment rarely occurred (Kitamura, 1982).

Observation of micronuclear changes

To test whether nuclear changes are induced by attachment to polystyrene, micronuclei of attaching cells were observed on preparations fixed by Carnoy's fixative (acetic acid/ethanol, 1:3) and stained with the Feulgen procedure.

RESULTS

P. caudatum

Only mating reactive cells of the *P. caudatum* stocks tested attached to Falcon 1007 polystyrene dishes. The attachment occurred only at the tips of ventral cilia, and attachment induced the first step of nuclear activation, *i.e.* early micronuclear migration (EMM). Unexpectedly, however, stock CHB showed a low ratio of attachment (about 20–30%) even when they were strongly mating reactive (Table I). The percentage of attached cells decreased to less than 10% within 5 min following introduction of cells to the dishes, suggesting a weak affinity of stock CHB cells for polystyrene surfaces. Three clones obtained from selfing progeny of stock CHB also showed a similar low percentage of attachment (9–23%) (Table I). However, it is uncertain whether stock CHB has a genetic defect in the mechanism of polystyrene attachment.

TABLE II

Attachment of cells to the surface of polystyrene dishes in P. multimicronucleatum, *syngen 2*

Stocks	Mating type	% Cells attached to the dishes	
		Non-reactive cells	Mating reactive cells
109	III	0 (0–4)	55 (44–66)
203	III	0 (0–4)	49 (42–57)
204	IV	1 (0.1–4)	52 (47–58)
103	IV	0 (0–4)	0 (0–4)
CH 104	III	0 (0–2)	48 (40–56)
CH 313	III	1 (0.1–2)	53 (44–63)
CH 100	III	0 (0–2)	45 (37–54)
CH 312	IV	0 (0–2)	52 (42–62)
CH 326	IV	0 (0–2)	36 (28–45)
CH 2	IV	0 (0–2)	52 (44–60)

Cells were washed with a solution containing 1 m*M* KCl, 1 m*M* $CaCl_2$, and 1 m*M* Tris-HCl, pH 7.1, and equilibrated in the same solution for 10 min at 20°C.

P. multimicronucleatum

Mating reactive-dependent attachment to polystyrene was also observed in all of the tested stocks of *P. multimicronucleatum,* but only about 50% of cells attached even though more than 90% of the cells expressed mating reactivity. Moreover, cells of one unusual stock (stock 103) did not attach. In all experiments cells were cultivated in a 0.3% grass infusion (cerophyl) buffered with 0.1% Na_2HPO_4. Therefore, one may suspect that the ability of cells to attach to polystyrene was influenced by their growth in grass infusion. To test for this possibility, cells of the four stocks used in a previous study (Kitamura and Steers, 1983) plus six additional stocks were cultured in lettuce medium and attachment was tested. Table II shows that mating reactive-dependent attachment is highly reproducible in all stocks of *P. multimicronucleatum* except for stock 103 which showed no attachment even when mating reactive. Mating reactive cells attained a maximum attachment plateau within the first min following their introduction to the dishes (Fig. 1B). Notwithstanding that more than 90% of the cells had strong mating reactivity, approximately 50% attached, almost the same percentages as obtained with cerophyl cultured cells. These results demonstrate that there is no marked difference in attachment ability due to these culture conditions.

P. tetraurelia and P. trichium

Attachment to polystyrene surface was tested in four stocks of *P. tetraurelia* and four stocks of *P. trichium,* syngen 1. All of them showed mating-reactive dependent attachment, whereas attachment occurred in less than 7% of non-reactive cells (Table III). In *P. tetraurelia* the percentage of maximum attachment averaged 44% even when stocks hr^d of mating type VII and VIII were used. These are highly mating reactive mutants derived from stock 51 (Sonneborn, 1974). This low percentage of attachment probably reflects the presence of mating non-reactive cells in the sample because about 20% of cells used to test for attachment were found to be undergoing autogamy, and autogamous cells are known not to express mating reactivity (Metz, 1954).

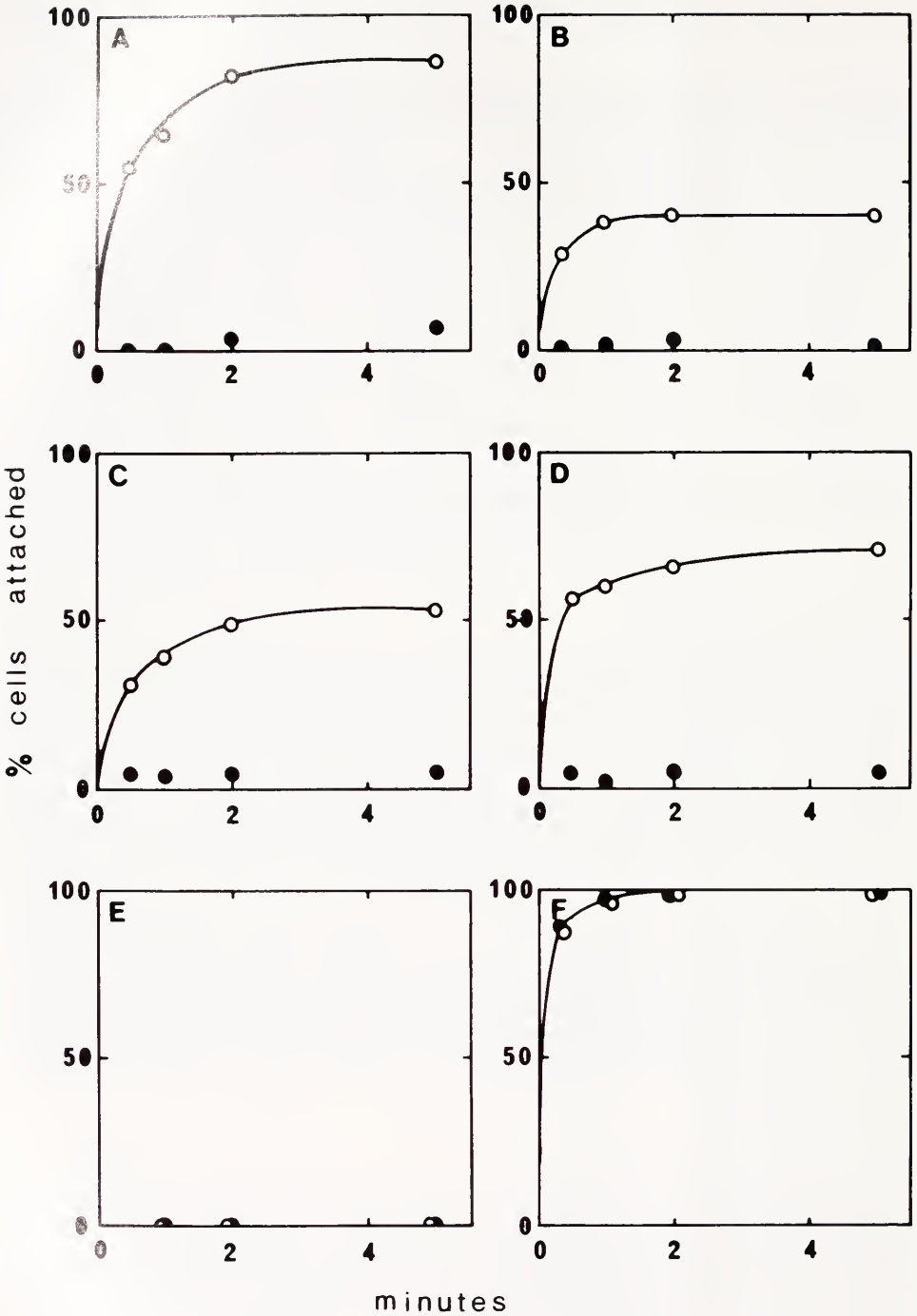

FIGURE 1. Typical adhesion kinetics of cells to the surfaces of polystyrene dishes in six species of *Paramecium*. Cells were adapted in glass tubes with a solution containing 1 m*M* KCl, 1 m*M* $CaCl_2$, and 1 m*M* Tris-HCl, pH 7.1, for 10 min at 21°C. After adaptation, they were transferred into Petri dishes to test for adhesiveness. A: *P. caudatum:* B; *P. multimicronucleatum:* C; *P. tetraurelia:* D; *P. trichium:* E; *P. bursaria:* F; *P. duboscqui.* Open circles = stationary phase cells with mating reactivity. Closed circles = mating non-reactive cells in log phase of growth.

TABLE III

Attachment of cells to the surface of polystyrene dishes in P. tetraurelia *and* P. trichium, *syngen 1*

Stocks	Mating type	% Cells attached to the dishes	
		Non-reactive cells	Mating reactive cells
P. tetraurelia			
hr^d	VII	4 (1–8)	39 (32–46)
hr^d	VIII	3 (1–5)	45 (38–53)
d4-186	VII	5 (2–9)	44 (38–51)
d4-186	VIII	4 (2–7)	48 (42–54)
P. trichium			
So 1	I	3 (0.5–5)	64 (56–71)
Sw 1	I	7 (5–11)	52 (45–59)
So 2	II	4 (1–8)	65 (58–72)
Sw 2	II	5 (3–8)	60 (54–66)

In contrast to *P. caudatum,* micronuclei in *P. tetraurelia* normally do not lie close to the macronucleus, and the obvious micronuclear movement corresponding to the EMM in *P. caudatum* is not present in normal conjugation. No marked change in micronuclear size or behavior was observed even 2 h following attachment.

In mating reactive *P. trichium* cells, a maximum attachment of about 60% occurs within one minute following introduction of the cells to the polystyrene dishes (Fig. 1D). Attachment was rarely seen in non-reactive cells.

In stationary phase cells of *P. trichium,* the micronucleus is normally closely associated with the macronucleus as in *P. caudatum.* Nonetheless, no changes in micronuclear size or behavior were induced in cells even after more than 4 h of attachment.

P. bursaria

Mating reactivity of *P. bursaria* syngen 1 appears only during the daytime in stationary phase cells. Cells that are in log phase of growth or in the night phase fail to show mating reactivity (Wichterman, 1948, 1953; Ehret, 1953; Cohen, 1964). Among the 17 natural stocks tested, attachment was rarely seen in either mating reactive or non-reactive cells (stationary phase cells in the night) during the first 5 min following the addition of cells to the dishes (Table IV, Fig. 1E) In addition to natural stocks, three F1 hybrid clones between stocks 492 and T81C2 also did not attach to polystyrene (data not shown). Attachment was not seen in non-reactive cells of other states such as log phase in the daytime or at night. However, a marked change in swimming behavior occurred about 10 min after the transfer of cells into the dishes. Cells stopped swimming and remained near the bottom of the dish. By 30 or 40 min, more than 80% of cells showed cessation of swimming. Some cells remained immobile at the surface of the medium. Although the tracks of such resting cells can not be easily distinguished on the photographs from those attached to polystyrene surfaces, the resting cells did not show a clear attachment to polystyrene. They sometimes showed creeping, though very slowly, along polystyrene surfaces. Similar cessation of swimming was seen when cells were applied to glass dishes. Neither symbiotic *Chlorella*-containing stocks nor stocks deprived of their symbiotic green algae showed attachment (Table IV). This indicates that the algae did not inhibit attachment. Attachment was not seen at 30°C or even when the swimming velocity of cells was reduced to 45% of the normal velocity by increasing the external KCl concentration to 15 m*M*.

TABLE IV

Attachment of cells to the surface of polystyrene dishes in P. bursaria, *syngen 1*

Stocks	Mating type	Geographical origin	% Cells attached to the dishes	
			Non-reactive[a]	Mating reactive[b]
So 11	I	Nagoya, Japan	1.6	3.3
Ih 1*	I	Iwate, Japan	3.9	0
Ok 1	I	Aichi, Japan	0	0
So 13	II	Nagoya, Japan	2.8	0
Cs 2	II	Shanghai, China	2.3	0.8
Ok 4	II	Aichi, Japan	5.3	5.1
M 4	II	Miyagi, Japan	9.6	2.4
F 36	II	Nagoya, Japan	0	0
F 29	III	Nagoya, Japan	0	0
T 157	III	Ibaraki, Japan	5.3	5.1
So 5	III	Aichi, Japan	1.1	0
T 151*	IV	Ibaraki, Japan	0.9	1.7
T 316	IV	Ibaraki, Japan	0.9	1.5
Osk 3	IV	Osaka, Japan	2.7	0
Osk 4	IV	Osaka, Japan	1.6	2.1
Nn 7*	IV	Niigata, Japan	0	0
Nn 8	IV	Niigata, Japan	0	0

* Symbiotic *Chlorella*-free strain.
[a] Stationary phase cells at night.
[b] Stationary phase cells in the daytime.

However, if cells were treated with the hydrophobic reagents 0.5 m*M* benzylamine or phenethylamine for 10 min, more than 80% of the cells showed attachment within 2 min after pouring cells into the dishes and more than 90% of the cells were still attached as much as 5 h later. About 40% of cells treated with trypsin (50 μg/ml) for 30 min also showed attachment. However, this effect of trypsin was obtained only in mating reactive cells even though their mating reactivity was completely destroyed by the treatment. Trypsinization of non-reactive cells did not induce attachment.

In normal conjugation of the *P. bursaria* stocks used in this study, micronuclear movement corresponding to the EMM found in *P. caudatum* was not observed. Before conjugation, most micronuclei lie near the macronucleus. The micronucleus increases in size about 5 h after onset of the mating reaction. However, when mating reactive cells were induced to attach to dishes by pre-treatment with 0.5 m*M* benzylamine, no nuclear changes were evident up to 6 h following attachment.

P. duboscqui

Unlike the other 5 species of *Paramecium* used in this study all 15 stocks of *P. duboscqui* tested showed attachment even when cells were non-reactive for mating (Table V). When the attachment process was examined with an interference microscope, the attachment was shown to occur first at the tips of the antero-ventral cilia of both mating reactive and non-reactive cells. The attachment kinetics of these two types show that more than 90% of the cells adhered to polystyrene within the first minute following introduction of the cells to the dishes (Fig. 1F). Photos of cell tracks taken at 1 h intervals showed that almost all of the cells were still attached more than 6 h later, and such cells showed normal swimming behavior when transfered to glass

TABLE V

Attachment of cells to the surface of polystyrene dishes in P. duboscqui

Stocks	Mating type	% Cells attached to the dishes	
		Non-reactive	Mating reactive
1a	I	98	100
1b	I	96	99
11	I	98	95
12	I	97	98
15	I	97	97
22	I	97	95
23	I	99	96
24	II	96	98
25	II	88	94
26	I	95	77
31	I	88	98
32	I	97	96
34	I	95	93
37	I	86	91
310	I	80	82

Cells were washed and suspended in a solution containing 1 m*M* KCl, 1 m*M* $CaCl_2$, and 1 m*M* Tris-HCl, pH 7.1, at 20°C. Ten min later, 5 ml of the cell suspension of each stock was introduced into polystyrene dishes.

Petri dishes with a micropipette. Strong affinity for polystyrene surfaces was also observed when cells were applied to Falcon 1001 or lids of Falcon 3002 dishes. These are less hydrophobic than Falcon 1007 dishes generally used in this study. More than 90% of cells showed attachment to both kinds of dishes regardless of mating reactivity.

No clear morphological or positional changes were observed in the micronucleus when cells were examined up to 5 h following attachment to polystyrene surfaces.

DISCUSSION

In this study, differences in adhesiveness of cells to polystyrene dishes were demonstrated among six species of *Paramecium*. These species can be classified into three groups based on their adhesiveness to polystyrene. Group I includes *P. caudatum, P. multimicronucleatum, P. tetraurelia,* and *P. trichium* which show mating reactive-dependent attachment. Group II is represented by *P. duboscqui* which shows mating reactive independent attachment. No attachment occurs in group III unless cells are pretreated with hydrophobic reagents as seen in *P. bursaria*. In group I, a considerable difference is found in the degree of adhesiveness among the four species. They can be arranged depending on the degree of attachment as follows; *P. caudatum* > *P. trichium* > *P. tetraurelia* ≃ *P. multimicronucleatum* (Tables I–III, and Fig. 1). However, as mentioned in the Results, the *P. tetraurelia* cell population contained some autogamous cells. These may not attach to the dishes due to lack of mating reactivity. Consequently, the percentage of attachment in mating reactive cells may be underestimated.

P. bursaria cells tend to stop their movement 10 to 30 min after transfer into dishes. Does this cessation of swimming indicate attachment to polystyrene surfaces? Iwatsuki and Naitoh (1979) reported that *P. bursaria* syngen 1 cells show thigmotaxis

5 to 30 min after applying them to glass dishes. Cells that attach to polystyrene surfaces usually do so upon initial contact with the surface and a marked inhibition of ciliary movement is seen (Kitamura, 1982; Kitamura and Hiwatashi, 1984), while collision of cells with solid surfaces induce thigmotaxis (Iwatsuki and Naitoh, 1979). Additionally, microscopical observations of the cells with thigmotaxis revealed that most cells were creeping along the surface. Therefore, those two phenomena are clearly distinguishable from one another.

It is uncertain whether there is a correlation between attachment ability and classifications based upon cell shape as described by Woodruff (1921). All 'aurelia' group species used, *i.e., P. caudatum, P. multimicronucleatum,* and *P. tetraurelia,* belong to attachment group I. However, *P. trichium* which belongs to the 'bursaria' group (III) also showed mating reactive-dependent attachment (Table III). It should be noted that *P. duboscqui* whose body shape is of intermediate size (Chatton and Brachon, 1933; Jin *et al.,* 1981) has a different type of attachment from 'aurelia' and 'bursaria' groups.

Other ciliates also fall into the three attachment groups defined above. For example, *Tetrahymena thermophilia* (Wolfe and Colby, 1981; Kitamura, unpub. obs.), *Blepharisma japonicum* (Kitamura, unpub.), and *Pseudomicrothorax dubius* (Peck, pers. comm.) belong to group II and *Euplotes octocarinatus* and *Paraurostyla weissei* belong to group III (Kitamura, unpub.). Ciliates which show attachment only when they are not mating reactive have not yet been found.

Unlike in *P. caudatum,* attachment in *P. tetraurelia, P. trichium,* and *P. duboscqui* failed to provoke any obvious changes in the micronucleus such as EMM or an increase in its size. These results were predictable since the EMM of normal conjugation is observed only in *P. caudatum.* Other obvious micronuclear changes are not seen during the initial step of conjugation in species other than *P. caudatum.* In those species a direct contact of cell bodies of polystyrene might be necessary for nuclear activation.

Finally the observation of different adhesiveness to polystyrene surfaces among six species of *Paramecium* suggests differences in hydrophobicity of ciliary membrane surfaces among them. Our further studies are directed toward biochemically analysing the components of their ciliary membranes.

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